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Hydrodynamic retrograde intrabiliary injection (HRII) in small (weaned) pigs for delivery of non-viral, naked DNA vectors for liver gene therapy

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Hydrodynamic retrograde intrabiliary injection (HRII) in small domestic (weaned) pigs for delivery method of non-viral, naked DNA vectors for liver gene therapy

Abstract

Gene delivery of therapeutic vectors remains challenging for *in vivo* gene therapy. All ongoing and approved human trials for liver gene therapy are based on adeno-associated viral vectors despite concerns about a future routine clinical use. Naked DNA vectors could be alternatives that have advantages over viral vectors, but delivery to hepatocytes remains a limiting factor. The aim was to establish naked DNA vector administration via hydrodynamic retrograde intrabiliary injection (HRII) in weaned pigs as large animal model for newborn pre-clinical application studies. The surgical procedure involved laparotomy and for vector delivery, a catheter was placed within the common bile duct by enterotomy. Optimal condition was found to be up to 12 mg of DNA vector in a volume of 100 ml and an infusion time of 10 sec, followed by 1 min clamping. No histological abnormalities were found in liver tissue upon pressurized injections. Stable transfection of up to 100% of hepatocytes with 12 mg of vectors expressing luciferase (at day 10; n = 6) was observed. In summary the HRII was less efficient, i.e. lower luciferase activity and vector copy numbers, than the intraportal injection delivery method (which was previously established in the same laboratory), but less stressful for the piglets, and has the potential for injection of vector DNA by the less invasive endoscopic retrograde cholangiopancreatography.

Keywords: pig, liver gene therapy, hydrodynamic injection, non-viral, DNA-vector

Vetsuisse-Fakultät Zürich 2020
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Hydrodynamische retrograde intrabiliäre Injektion (HRII) in jungen domestizierten (abgesetzten) Schweinen, als potenzieller Leber-Gentherapieansatz mit nicht-viraler, nackter DNA als Vektor

Zusammenfassung

Die Einschleusung von therapeutischen Vektoren bleibt eine Herausforderung für die *in vivo* Gentherapie. Humanstudien und Anwendungen für die Leber-Gentherapie basieren auf Adeno-assoziierten viralen Vektoren, trotz Bedenken klinischer Routineanwendungen. Alternativ könnten nackte DNS-Vektoren dienen, die Vorteile gegenüber viralen Vektoren aufweisen, jedoch die Einschleusung in Hepatozyten als limitierenden Faktor haben. Ziel war die Etablierung der Übermittlung von Vektoren in Absetzferkeln durch hydrodynamische retrograde intrabiliäre Injektion (HRII). Der chirurgische Eingriff beinhaltete eine Laparotomie und die Einschleusung erfolgte durch einen in den Gallengang gelegten Katheter mittels Enterotomie. Als optimal erwies sich bis zu 12 mg Vektor in 100 ml und einer Infusionszeit von 10 Sekunden, gefolgt von einer 1-minütigen Klemmung. Keine histologischen Veränderungen wurden nach Injektion festgestellt. Eine stabile Hepatozyten-Transfektion von bis zu 100% mit 12 mg Luciferase exprimierenden Vektor (an Tag 10; n = 6) wurde beobachtet. Zusammenfassend war die HRII weniger effizient, d.h. geringere Luciferaseaktivität und Vektorkopienzahlen als die intraportale Injektion (welche ebenfalls in unserem Labor etabliert wurde), jedoch weniger belastend für die Ferkel und weist das Potenzial für die Injektion durch die weniger invasive endoskopische retrograde Cholangiopankreatographie auf.

Schlüsselwörter: Schwein, Leber-Gentherapie, hydrodynamische Injektion, nicht-viral, DNS-Vektor

Abbreviations

AAV	adeno-associated virus
EDTA	ethylendiaminetetraacetic acid
ERCP	endoscopic retrograde cholangiopancreatographie
HRII	hydrodynamic retrograde intrabiliary injection
IM	intramuscular
IV	intravenous
MC	minicircle
PCR	polymerase chain reaction
RLU	relative light unit
TEM	transmission electron microscopy
UH	Dr. med. vet., Dr. rer. nat. Udo Hetzel, FTA (Pathologist)

1. Introduction

The overall goal of gene therapy is the permanent correction of an inherited or acquired genetic defect. Today, the most common gene therapeutic approach is gene addition, i.e. to provide a template for expression and thus replacement of a defective protein. A big advantage of gene therapy is that inborn as well as acquired diseases can be treated and for instance liver-directed gene therapy could replace liver transplantation in various monogenic liver disorders [1–4]. For *in vivo* gene delivery, the leading candidate today is the adeno-associated virus (AAV) vector with a high efficiency of transduction for a broad range of target tissues [4,5]. Clinical improvement of therapeutic AAV-based vectors to the retina, liver, and nervous system could be shown in patients with congenital disorders (e.g. blindness, haemophilia B, and spinal muscular atrophy) [6]. Gene therapy can be achieved by three approaches: gene addition, gene editing and gene knockdown [2]. As of today, the most clinical trials for gene therapy take place in the United States of America (61.2%) followed by Europe (22.7%) and Asia (11.1%). The distribution in Europe is as follows: 7.5% in UK (228 trials), 3.5% in Germany (106 trials), 2.0% in France (62 trials), and 1.7% in Switzerland (50 trials). The majority of gene therapy clinical trials have focused on cancer (66.6%) and monogenic diseases (11.9%). The most popular vectors are viral vectors, including adenovirus (18.5%, 575 trials) and retrovirus (16.9%, 525 trials), followed by non-viral naked/plasmid DNA vectors (15.0%, 466 trials) (http://www.abedia.com/wiley/ October 2020).

An alternative to viral vectors for *in vivo* gene therapy is the physical delivery of naked DNA. Although primarily used as an experimental approach in mouse studies, hydrodynamic injection of DNA into the liver is an established delivery method to treat inherited metabolic diseases [4,6]. Non-integrating, non-viral vectors such as naked DNA are an alternative to overcome certain disadvantages, e.g. immune response against viral capsids or possible mutagenesis by integration of viral vectors. Administration or delivery is challenging because naked DNA is fragile and requires a sufficient transfection rate to be expressed. Technical and chemical limitations are the efficiency of vector transport, unloading into cells and their transfection efficacy into the nucleus [1,7]. However most of them resulted in low and transient gene expression in the liver [8]. Pressurized (hydrodynamic) injections were described first in 1999 in murine models and resulted in positive hepatocyte transfection [9,10]. Successful long-term treatment via hydrodynamic tail vein injection with minicircle-based naked DNA vectors could be shown for Phenylketonuria and Cystathionine β-synthase deficiency in mice [11–13]. This technique is feasible and very efficient in mice but less

efficient in large animal models where one has to target the liver more directly [14]. Despite that, different techniques of administration have been established to deliver those vectors in an efficient way. Successful and feasible methods to target hepatocytes could be shown via the jugular vein (anterograde), via the portal vein (retrograde), via endoscopy (minimal invasive) in pigs, and intrabiliary (retrograde) in rats [15–19].

As a prerequisite to treat newborn or infant patients with inherited metabolic disorders of the liver, the Metabolic Unit of the University Children's Hospital Zurich (Medical Faculty of the University of Zurich) in collaboration with the Vetsuisse Faculty of the University of Zurich has previously established liver transduction in small pigs (after weaning) by hydrodynamic injection of naked DNA vectors via the portal vein [17]. These circular DNA vectors, also called minicircles, contained an expression cassette with luciferase as marker gene under the control by a liver-specific promoter [11]. Minicircles are devoid of any bacterial plasmid DNA backbone and allow sustained transgene expression in quiescent cells and tissues, and remain in the genome as episomal elements. Minicircle DNA vectors are propagated in a genetically modified *Escherichia coli* strain and purified thereafter by conventional plasmid-DNA methods (see **Figure 1**) [11,13,20]. While pigs suffered substantially from surgical stress induced by portal vein catheterization, stable hepatocyte transfection was found up to 60% in liver samples (45 out of 75) PCR-positive for vector-DNA and 13% of these positive specimen (6 out of 45) with stable luciferase expression [10]. In follow-up experiments applying identical physical and surgical conditions for intraportal injection, the vector DNA dose was increased 6-fold, i.e. from 2 mg to 12 mg of MC.P3-luc. This resulted in 100% of samples to be positive for vector-DNA by PCR (75 out of 75) and 75% of these positive specimen were found with stable luciferase expression (56 out of 75; personal communication of unpublished observations). Despite this significant improvement of liver transduction via the portal vein, we sought for an alternative and safer route for physical delivery of vector DNA into hepatocytes.

Injection of a solution containing viral vectors, naked DNA or nanoparticles for liver targeting via the common bile duct was reported in mice, rats, dogs, (adult) pigs but was never described in small pigs after weaning [15,18,19]. Moreover, pressurized infusion into small pigs via the bile duct was not described.

The aim of this medical veterinary thesis was to evaluate and characterize the intrabiliary injection route for naked DNA vectors into small pigs by open surgery as an alternative and potentially safer way for (hydrodynamic) gene delivery to hepatocytes than the intraportal vein injection. As DNA vector, we used our standard MC.P3-luc vector, expressing the luciferase from the liver-specific promoter P3. Furthermore, we hypothesized that retrograde intrabiliary entry allows to "circumvent" the sinusoidal endothelial cells, an anatomical structure and potential barrier which might be a limiting factor for DNA delivery to hepatocyte through the portal vein [21,22]. Moreover, once established, the retrograde intrabiliary injection route might eventually be amenable to humans by endoscopic retrograde cholangiopancreatography (ERCP), which is an established method that allows physical access to liver cells via the bile duct.

2. Materials and Methods

2.1 Naked DNA/minicircle (MC) vectors

The purified MC.P3-luc (2324 bp) vector was used that expresses the firefly luciferase gene from the liver-specific-promoter P3 [11,20,23] (**Figure 1**). Vector DNA was purified by using a commercial kit, i.e. Macherey-Nagel™ NucleoBond™Xtra Maxi EF.

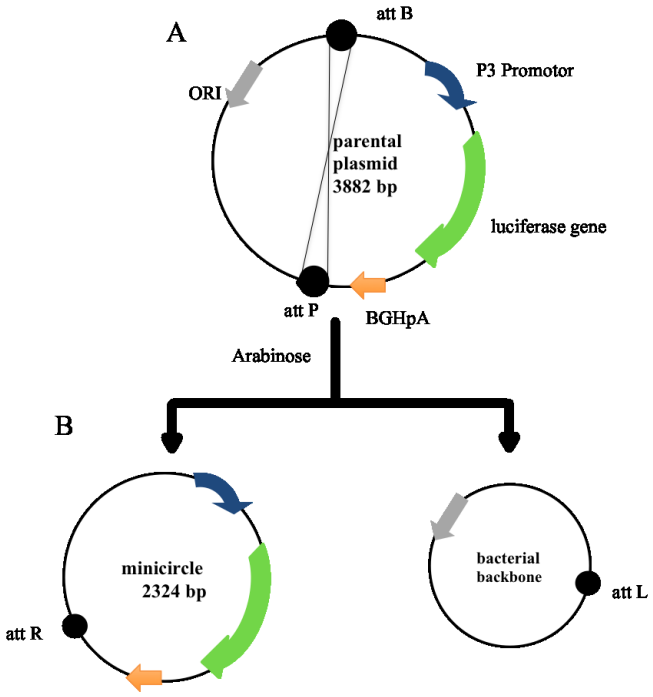


Figure 1: *Generation and harbouring of Arabinose-induced minicircles. (A)* Parental plasmid was transformed into the bacterial strain ZYCY10P3S2T, expressing integrase and endonuclease, both under the control of the arabinose-inducible promoter p*BAD* [20]. (B) The minicircles are generated via intramolecular recombination after adding arabinose. The excised bacterial backbone is degraded *in vivo*. The harboured minicircle contains a liver specific P3 promotor for an expression only in hepatocytes, luciferase as a reporter gene, and a BGHpA element.

2.2 Animal Handling

Animal experiments were performed according to the guidelines and policies of the Veterinary Office at the State of Zurich and Swiss law on animal protection, the Swiss Federal Act on Animal Protection (1978), and the Swiss Animal Protection Ordinance (1981). Animal studies were approved by the Veterinary Office, Zurich, and the Cantonal Committee for Animal Experiments, Zurich, Switzerland (permission animal experiments Kt ZH 176-2016). Three-week-old female domestic pigs (weight 5.3–6.4 kg; ***Table 2***) were separated from their mothers after weaning and brought to a loose barn with porcine mates 5 days before surgery.

2.3 Anaesthesia and postoperative monitoring

Pre-anaesthetic medication: Azaperone 2 mg/kg IM, Ketamine 5mg/kg IM and Atropine 0.03 mg/kg IM, induction: isoflurane mask, maintenance: isoflurane and oxygen. Animals were kept for 6 hours, 3 or 10 days (day 9 or day 11 if the day of sacrifice fell on a weekend) after surgical intervention. Liver transaminases (aspartate aminotransferase, AST; alanine aminotransferase, AST) and lactate dehydrogenase (LDH) were measured in the Unit for Clinical Chemistry and Biochemistry at University Children's Hospital Zurich, Zurich, Switzerland, by automated analyzer UniCel DXC600 (Beckman Coulter, Nyon, Switzerland) at day of surgery and day of sacrifice. Plasma, cerebrospinal fluids (CSF) and urine were stored shock-frozen for any later analyses. The following medication was used for postoperative care: Buprenorphine 0.01 mg/kg IV TID and Enrofloxacin 5% 2.5 mg/kg IM once daily. In case of fever, Metamizole 25 mg/kg IV was added. Anaesthesia equipment and instruments are shown in **Figure 2**.

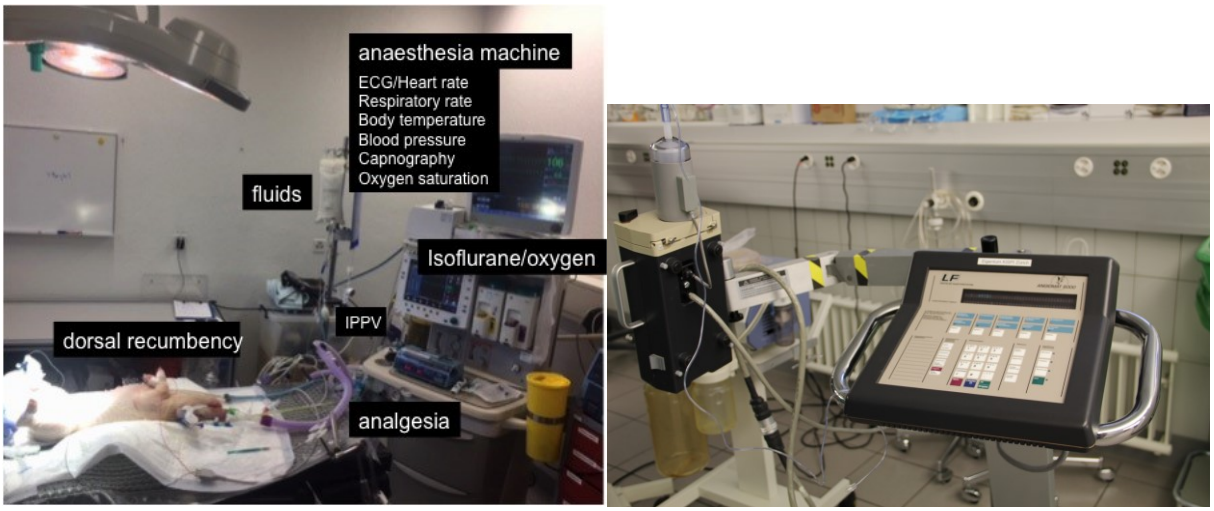


Figure 2: *Anaesthesia set-up.* On the left picture: the patient is in dorsal recumbent position and on a closed circuit anaesthesia system. Heart rate was monitored via ECG, respiratory rate via IPPV (intermittent positive pressure ventilation), blood pressure via an arterial catheter (A. femoralis), and body temperature via oesophageal thermometer. The capnography monitor shows the concentration of carbon dioxide in the respiratory gases. As inhalation gas was isoflurane used and for ventilation a mixture of oxygen and air. Infusion pumps for controlled analgesia. On the right picture: A contrast injector, Liebel-Flarsheim Angiomat 6000, which allowed us to inject naked DNA via pressure. The control panel allowed us to adjust pressure, volume, and flow rate.

2.4 Surgical procedure for hydrodynamic retrograde intrabiliary injection (HRII)

Laparotomy was performed from xyphoid to the umbilicus, followed by isolation/separation of the hepatic arteries of the hepatic ligament. The portal vein was prepared distal from the

hepatic ligament and separated from the pancreas over a distance of 1 cm to place a clamp. Then, a 2 cm longitudinal enterotomy was performed anti-mesenterial next to the insertion of the common bile duct into the duodenum. A 7 Fr single-lumen catheter was placed through the major duodenal papilla in the common bile duct. The position of the catheter was initially monitored by injecting contrast medium (pre- and post-injection; Accupaque ® 300 mg, GE Healthcare AG, Glattbrugg, Switzerland) via Real-Time XRay (***Figure 3*****B–E**). Once established, we injected the catheter without contrast medium and ensured manually that the tip of the catheter was placed into the common hepatic duct 1 cm before the hepatic porta. This allowed us a controlled injection of naked DNA-/MC-vectors (between 2 – 12 mg naked DNA diluted in a volume of 100 ml Ringer's solution injected within 10 sec. according to ***Table 2***) into the entire liver by using a Liebel-Flarsheim Angiomat 6000 (2111 E. Galbraith Rd. Cincinnati, Ohio 45215, United States of America) (**Figure 2**). The catheter was tightly fixed with two clamps (Atraumata® Bulldog Clamps, FB364R, FB366R), which also temporarily closed the enterotomy. For all experiments, clamps were placed at the cystic duct to avoid backflow to the gallbladder, to the hepatic arteries and the portal vein allowing hepatic inflow obstruction (***Figure 3*****A**). Likewise, the caudal cava vein was clamped in order to prevent venous outflow from the liver during the injections [17,24]. The total clamping time was kept short, i.e. no longer than 1-2 min given the known vulnerability of the pig intestine to outflow obstruction. After injection, all clamps were removed in the following order: caudal cava vein, portal vein, hepatic arteries and cystic duct. The catheter was removed and the enterotomy was closed with absorbable running suture and patency and tightness were tested. Urine was collected for analysis by needle puncture of the bladder, and thereafter the abdominal cavity was flushed with ringer lactate to reduce contamination. The abdominal cavity was closed stepwise (muscle layer, subcuticular layer) with absorbable running sutures. The wound was covered with standard dressing.

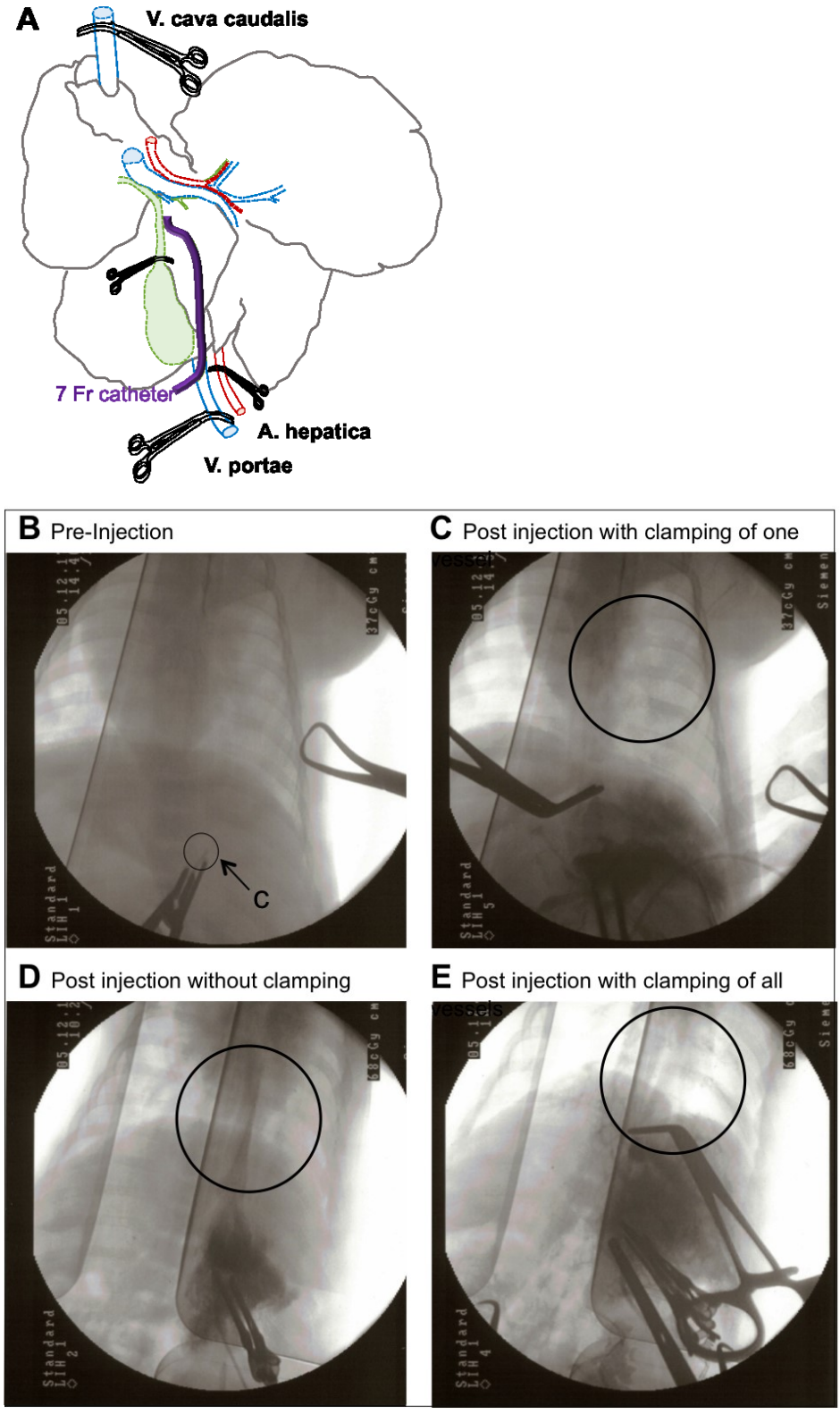


Figure 3: *Scheme of the hydrodynamic intrabiliary infusion (HRII) and outflow examination via Real-Time X-Ray.* **(A)** Scheme of a pig liver with V. portae, A. hepatica, V. cava caudalis, and the gallbladder including biliary tract. Laparotomy and transient clamping (black clamps) of V. portae, A. hepatica, V. cava caudalis, and additionally the cystic duct (asterisk) were performed to target hepatocytes in the periportal area. Access of the HRII procedure was performed by enterotomy and moving the catheter forward through the papilla duodeni major (not shown) into the common hepatic duct (black arrow). The position of the catheter was ensured and tightly fixed with clamps. **(B)** Pre-injection to check catheter position (circle with black arrow indicates the tip of the catheter). **(C)** Post contrast medium injection while obstructing Vena cava caudalis: no outflow visible

(circle). **(D)** Post injection of contrast medium (22 ml NaCl plus 8 ml contrast medium) without clamping: outflow in direction to the heart visible (circle). **(E)** Post-injection of contrast medium with clamping of all three vessels (c): no outflow visible (circle).

2.5 Collection of samples

Blood and urine samples for various biochemical analyses and for naked DNA-/MC-vector determination were collected at the day of surgery and at the day of sacrifice. Pigs were sacrificed under deep anesthesia with a combination of Xylazine and Ketamine and released with Pentobarbital 6 hours, 3 days or 10 days after following injection. The entire liver was resected to collect 75 individual tissue samples that represent all 5 liver lobes (***Figure 4***). In addition, samples from heart, spleen, diaphragm, lung, and kidneys were collected and stored shock-frozen.

Cryo sections, hematoxylin and eosin staining and immunohistology

For histological evaluation, liver tissue from representative lobes was either snap frozen immediately for cryo sectioning (3–5 µm, PrestoCHILL, Kalamazoo, MI 49009, USA) for demonstration of hepatocellular lipid (Oil-Red-O), or fixed in formalin (4 %), and routinely paraffin embedded. Histological sections (3–5 µm) were prepared and routinely stained for hematoxylin and eosin (HE) and Periodic acid-Shiff (PAS) reaction, the latter to demonstrate hepatocellular glycogen content.

Immunohistological evaluation was performed on cryo sections to detect luciferase positive cells using anti-Luciferase antibody (Abcam, ab 181640) with Dako autostainer (Dako, Glostrup, Denmark) system. Briefly, antigen retrieval was performed in the antibody using citrate buffer (pH 6) at 98°C for 20 min. or EDTA buffer (pH 9) at 98°C for 20 min. and CC1 buffer (pH 8.4). Subsequently, endogenous peroxidase activity was quenched with hydrogen peroxidase for 10 min. Primary antibodies were incubated for 1 hour followed by secondary antibody application. For detection, the Dako EnVision kit (Dako, Glostrup, Denmark) was used. Finally, sections were counterstained with hematoxylin for 40 sec. and mounted. Luciferase positive pig liver served as positive control for luciferase antibody. As negative controls served sections omitting the primary antibody. Specimens were evaluated twice by one pathologist (UH, Vetsuisse Faculty Zurich).

Transmission electron microscopy (TEM)

Samples of liver for TEM evaluation were trimmed immediately after liver exenteration into 1 mm square blocks and fixed in 2.5 % glutaraldehyde (EMS) buffered in 0.1 M Na-P-buffer

overnight, washed x3 in 0.1 M buffer, post fixed in 1 % osmium tetroxide (Sigma-Aldrich) and dehydrated in ascending concentrations of ethanol followed by propylene oxide and infiltration in 30 % and 50 % Epon (Sigma-Aldrich). At least three 0.9 µm toluidine blue stained semi-thin sections per localization were produced. Representative areas were trimmed and 90 nm, lead citrate (Merck) and uranyl acetate (Merck) contrasted ultrathin sections were produced and viewed under Phillips CM10 operating with Gatan Orius Sc1000 (832) digital camera, Gatan Microscopical Suite, Digital Micrograph, Version 230.540. All samples were processed by the pathological laboratory (under the pathologist UH) of the Vetsuisse Faculty Zurich.

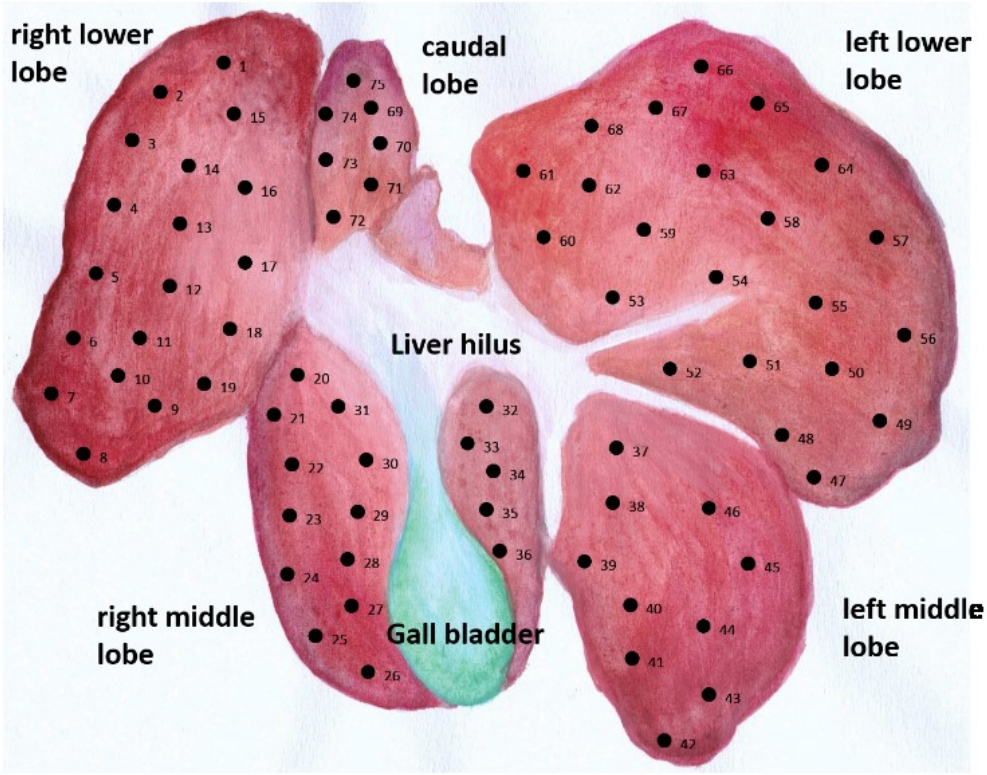


Figure 4: *Anatomical sketch of the five pig liver lobes and the distribution of all 75 collected samples.* Samples were collected and snap-frozen immediately and/or fixed in formalin (4%) after resection.

2.6 MC vector-DNA analysis in pig liver

For liver tissue homogenization, the TissueLyser II (Qiagen, Hombrechtikon, Switzerland) was used. DNA extraction from liver homogenates was performed according to the DNeasy Blood and Tissue Kit (Qiagen GmbH, Germany). Purity and quality of DNA was determined with the NanoDrop ND1000 (Thermo Fisher Scientific). Saturating PCR for detection of

vector DNA was performed from isolated tissue samples. As forward primer 5'-CAC GTT CGT CAC ATC TCA TCT ACC-3' (primer f2 luc, Microsynth AG, Balgach, Switzerland) and as reverse primer 5'-TGA GCC CAT ATC CTT GCC TGA TAC-3' (primer r3 luc, Microsynth AG, Balgach, Switzerland) were used to obtain and amplify MC.P3-luc. PCR for 42 cycles using HOT FIREPol polymerase (Solis BioDyne, Lucerne, Switzerland) was done at a denaturation temperature of 95°C, an annealing temperature of 63°C, and an extension temperature of 72°C. The amplified fragment had an expected length of 533 bp. As positive control, isolated DNA from wild-type mouse liver after hydrodynamic tail vein injection with MC.P3-luc, and as negative control, isolated DNA from untreated/non-infused pig liver was used.

2.7 Assay for MC Copy Number

Genomic DNA (gDNA) from all 75 liver samples was isolated by using DNeasy blood and tissue kit (Qiagen GmbH, Germany). According to the manufacturer's manual, we used 100 ng of gDNA from each sample as a template. Serially diluted DNA vector with various copy numbers (2x107 copies to 20 copies) for MC.P3-luc, along with 100 ng non-infused control gDNA; were generated to plot standard curves cycle threshold (Ct; y axis) against log vector copy number (x axis) for each vector infused in pig livers (standard curves for all pigs see **Table 1**). To determine the number of vector genomes per cell in liver tissue, we performed absolute quantitative PCR (qPCR) analysis by either using TaqMan gene expression assay corresponding to luciferase (Mr03987587_mr, Life Technologies) or primers and probe bind to BGHpA in all liver tissue samples. Used sequences for the primers and the probe, as forward primer 5'-GCC TTC TAG TTG CCA GCC AT-3', as reverse primer 5'-GGC ACC TTC CAG GGT CAA G-3', and as probe 5'-TGT TTG CCC CTC CCC CGT GC-3'. For qPCR measurement we used ABI PRISM 7900 sequence detector, and for analysis Sequence Detection System (Life Technologies). The haploid genome size of pig is estimated to be 2800 Mb (according to NCBI Genome Database) and the mass of a single diploid copy is 6.14 pg (calculated according to the description from Life Technologies). Consequently, 100 ng gDNA contains 16,287 copies of diploid genome (1x105 pg/6.14 pg).

***Table 1**: standard curves of all pigs for MC.P3-luc[1] and nSMARter.P3Luc1[2]*

Pig no	standard curves
176-25[1]	no vector injected
176-26[1]	y=-3.8x + 40.6, R2=0.996 and y=-3.9x + 40.8 R2=0.996
176-27[1]	n.d.
176-28[1]	y=-3.4x + 38.2, R2=0.999 and y=-3.6x + 38.3, R2=0.999
176-29[1]	y=-3.4x + 37.9, R2=0.995 and y=-3.4x + 36.7, R2=0.997
176-30[1]	y=-3.2x + 35.9, R2=0.999 and y=-3.3x + 36.6, R2=0.999
176-31[1]	y=-3.2x + 36.9, R2=0.999 and y=-3.3x + 36.1, R2=0.999
176-32[2]	y=-3.4x + 38.4, R2=0.998 and y=-3.3x + 38.4, R2=0.998
176-33[2]	y=-3.4x + 39.1, R2=0.999 and y=-3.4x + 39.0 R2=0.998
176-34[2]	n.d.
176-35[1]	n.d.
176-36[2]	y=-3.3x + 41.1, R2=0.998 and y=-3.2x + 40.5 R2=0.997
176-37[1]	y=-3.4x + 39.6, R2=0.999 and y=-3.4x + 39.3 R2=0.998

n.d., not determined (due to liver rupture or general poor health conditions and early euthanasia)

2.8 Luciferase activity

Luciferase activity was measured in liver tissue samples to study the expression levels of injected naked DNA. Therefore, µg per µl lysate was measured and divided by RLU (relative light unit) per µg. The threshold defines as above or equals 0.08 RLU/µg Protein. Here, we used the Luciferase Assay System (Promega, Dübendorf, Switzerland) on the microplate reader infinite F 200 (Tecan Group Ltd., Männedorf, Switzerland). For data analysis, i-control 1.10 software (Tecan Group Ltd., Männedorf, Switzerland) was used. As positive control, liver tissue lysates from MC.P3-luc injected mice with MC expressing luciferase were used, and as negative control, a liver tissue lysate of an untreated/non-infused pig was taken.

3. Results

3.1 Establishment of the surgical procedure for HRII in piglets

Hydrodynamic portal vein injection was studied and established in the same laboratory in small pigs after weaning [17]. Although this injection method was successful but highly invasive and stressful, we thought to establish a less invasive method for the newborn pig. Intrabiliary injection was chosen for delivering a solution containing naked DNA vectors. Optimal injection parameters for portal vein injections had a flow rate of either 10 ml or 20 ml/sec. and an injection time between 1 and 3 sec., in a total volume of 30 ml with a maximal pressure of 120 mmHg [17]. Surgical set-up, placement of the catheter in the common bile duct, for intrabiliary injection was monitored and documented by Real-Time XRay (pig. no. 176-27, data not shown due to lack of further analyses). Here we observed, as expected, outflow into the heart without clamping (***Figure 3*D**) while clamping of all three vessels and the cystic duct (not shown) resulted in efficient sealing (***Figure 3*E**). Intraoperative (minor) complications such as bile duct or liver rupture could be observed, but were fixed and the piglets recovered normally after surgery. Again, the liver expanded transiently and went back to normal within seconds after opening the clamps. All hemodynamic parameters, especially during hydrodynamic injections and clamping, were carefully monitored. Piglets returned to a loose barn with porcine mates within 3–4 hours after end of anaesthesia. Surgical procedures combined with sham infusions were established in pig no A2. Sham infusion of 30 ml/10sec. with sterile saline solution was performed, followed by an injection of 100 ml/10sec. containing methylene blue as dye (***Figure 5***), followed by 1 min. of clamping. Expansion and reaction of the liver during open surgery was monitored. Liver biopsies for immunohistochemical analyses were collected before and immediately after infusion, and thereafter the animal's health was monitored for 10 days. Histologically no dye could be detected even immediately after infusion or after 10 days. According to the findings, the results of intraportal injection, and injection recommendations [18,25] we investigated in an impact study to determine integrity and mechanical damage to liver tissue upon pressurized injection (pig A1, ***Figure 5***). Therefore, some calculations about relation of pressure and diameter were performed (Bernoulli's principle, [26]). The pressure ratio change is quadratic to the volume flow. Two parameters were chosen for the impact study, a reduced flow rate 30 ml/10sec. (10-fold lower pressure) and our classic infusion (100 ml/10sec.), always followed by 1 min of clamping. In both conditions liver biopsies pre- and after injection were taken. No morphological damage could be observed either after a flow rate of 100 ml/10sec. or a 10-fold

lower injection rate (30 ml/10sec.) on HE staining, PAS staining, Oil-Red O or TEM. Histological investigations to exclude mechanical liver damage upon these two conditions were performed. The classical and the pressure reduced flow did not show any morphological changes or abnormalities on hepatocytes in HE (detection for necrosis), PAS, Oil-Red-O (demonstration of hepatocellular lipids) staining's (***Figure 6***) and in TEM (***Figure 7***).

3.2 Hepatic delivery of MC-DNA expressing luciferase to small pigs by HRII

According to the findings of the pigs no. 176-25, A1 and A2, as well as the experience, and the published results of intraportal injection [15–17,27], we considered that these conditions (sham infusion 30 ml/10sec., classic infusion 100 ml/10sec.) are optimal for HRII (***Figure 5***). Furthermore, we investigated in the infusion of naked DNA at different time points (6 hours, 3 days and 10 days after injection) as well as different dosages (2 or 12 mg of vector DNA).

Next, we injected into two individual pigs 12 mg of naked DNA vector, either MC.P3-luc (pig no. B1) or nSMARter.P3.luc1 (pig no. C2, see supplement) diluted in 100 ml of sterile saline solution injected within 10 sec. through the bile ducts, followed by 1 min. addition clamping. Time point of sacrifice was 6 hours after injection. In another pig (B3), 12 mg of MC.P3-luc was injected with the same conditions but sacrifice 3 days after injection. The next six piglets were injected with either 2 mg of MC.P3-luc (pig no. B4 and B5), 12 mg of MC.P3-luc (pig no. B6 and B7) or 12 mg of nSMARter.P3.luc1 (pig no. C3 and C4). Injection conditions were always our optimized protocol for HRII exception pig no C4, here was a 10-fold lower pressure used. Time of sacrifice was in all six piglets 10 days after injection (***Table 2***).

The result of PCR positive liver samples and copy number is increasing in relation to the dose (***Figure 8***, ***Table 3***). We found what we expected and observed with the HRII procedure a similar copy number as with the portal vein injection of 2 mg of MC.P3-luc vector. Luciferase expression upon HRII is comparable with the portal vein injection. The only difference we observed was the distribution of Luciferase positive samples, either on the right liver area (HRII) or on the left liver area (portal vein). Unexpectedly, titration of vector DNA up to 12 mg did not result in a higher hepatocyte transfection rate. A highest transfection rate could be observed in pig B1 (sample no. 51, 1.40 RLU/µg), which was sacrificed 6 hours upon HRII. Intraoperative leakage (bile duct/liver rupture) could explain the variation of the PCR results of pig no. B4 and B5 with the same amount of injected DNA vectors. In pig no. B5 less than 2 mg of the infused vector solution were administered. Another reason therefore could be the allometry of pigs. It is known that the skeleton, the muscles and organs grow at different rates

of growth, i.e. the growth of the liver reaches 50% of the mature weight at the age of 12-14 weeks [28,29].

Only for one animal, we observed major post-operative complications that led to preterm euthanasia. This occurred due to wound infection, which was not related to the DNA infusion itself (pig no. 176-27, day 3; data not shown). In summary, all piglets (n = 8+3; see supplement) were stable intraoperative and did not show any adverse effects which let us conclude that the HRII technique is superior over the intraportal route of injection, as we observed less stress, mortality and a fast recovery of the pigs after surgery.

Liver transaminases (AST and ALT) and LDH were measured before, during the surgery, and at day of sacrifice (6 hours, Day 3, or Day 10). All values were slightly increased after the surgery but turned back to normal at day of sacrifice. This observation, data not shown, confirmed that there is no liver damage due to hydrodynamic infusion, as also seen by others [15,17,24].

Table 2: Characteristics of pigs infused with vector DNA by intrabiliary injection.

Pig no. and group (pig identity)[2]	Body weight on surgery day (kg)	Time of sacrifice and sample procurement[1]	Body weight on day of sacrifice (kg)	Liver weight on day of sacrifice (g)	Injected vector (mg)[2]	Flow rate (ml/sec); Injection time (sec) (total volume in ml)	Remarks
A1 (176-35)	4.7	6 h	6.9	300	---	10; 10 (100)	**Figure 3**
A2 (176-25)	5.2	10 d	6.4	350	---	10; 10 (100)	**Figure 6 and Figure 7**
B1 (176-37)	5.0	6 h	5.0	200	12	10; 10 (100)	**Figure 8A**
B3 (176-31)	5.7	3 d	6.9	300	12	10; 10 (100)	**Figure 8B**
B4 (176-26)	5.4	10 d	6.5	n.d.	2	10; 10 (100)	**Figure 8C**
B5 (176-28)	6.3	10 d	9.6	400	2	10; 10 (100)	**Figure 8D**
B6 (176-29)	6.4	10 d	9.1	300	12	10; 10 (100)	**Figure 8E**
B7 (176-30)	5.3	10 d	7.2	300	12	10; 10 (100)	**Figure 8F**
C2 (176-36)	6.7	6 h	6.7	n.d.	12	10; 10 (100)	**Figure 9A**
C3 (176-32)	5.4	10 d	7.2	340	12	10; 10 (100)	**Figure 9B**
C4 (176-33)	5.2	10 d	7.0	380	12	3; 10 (30)	**Figure 9C**

n.a., no data available

[1] days (d) or hours (h); note that "10 days" is between 9 to 11 days after injection

[2] group A and B pigs were injected with vector MC.P3-luc while group C pigs were injected with vector nSMARter.Luc1

Figure 5: *Overview plan of the infusion procedure for pressurized injection.* **A.** Impact study of pig A1 to observe damage to the liver tissue. Biopsies (red mark) were taken pre-injection and after two infusion conditions. 30 ml/10sec. (3 ml/sec.; 10-fold lower pressure) and 100ml/10sec. (10ml/sec.). Sterile saline solution in total volume of either 30ml or 100 ml was injected. The animal was sacrificed 6 hours after injection. **B.** Injection repetition without vector in pig A2 and sacrificed 10 days after injection. Biopsies (red mark) for histological analyses were taken after 100ml/10sec and 10 days after injection. No morphological abnormalities were observed (data not shown). **C.** Optimized for HRII: sham infusion 30ml/10sec., followed by pressurized injection of 100 ml/10sec. with vector (2 mg or 12 mg of MC.P3-luc or nSMARter.P3Luc1). The animals were sacrificed 6 hours after, on day 3, or day 10 after injection. Sterile saline solution was used for infusions without vector.

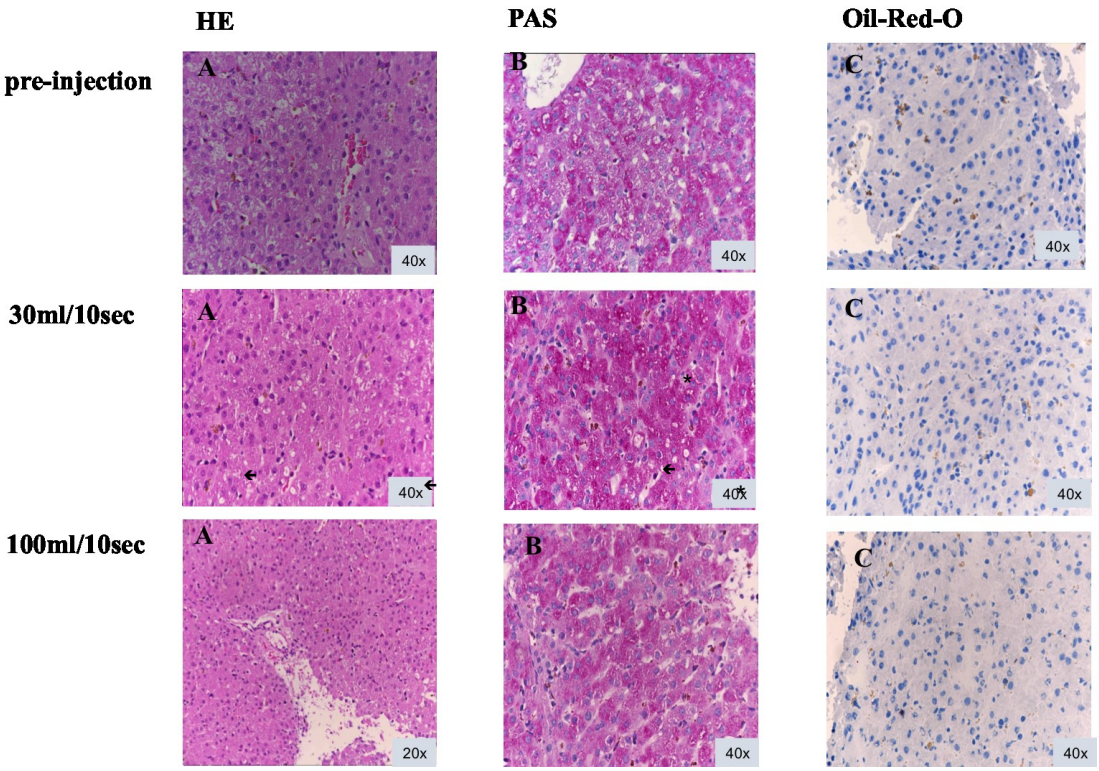


Figure 6: *Histological analyses of pressurized infusion to the liver tissue (pig A1).* Liver biopsies pre-injection and after two injection conditions (30 ml/10sec. and 100 ml/10sec.) of sterile saline solution were taken. Mild haemosiderosis (black arrows) within in normal limits could be detected in all three liver biopsies and cytoplasmatic vacuolation (asterisk) was detected after pressurized injection, but no morphological damage could be observed. A: HE staining, B: PAS staining, C: Oil-Red O.

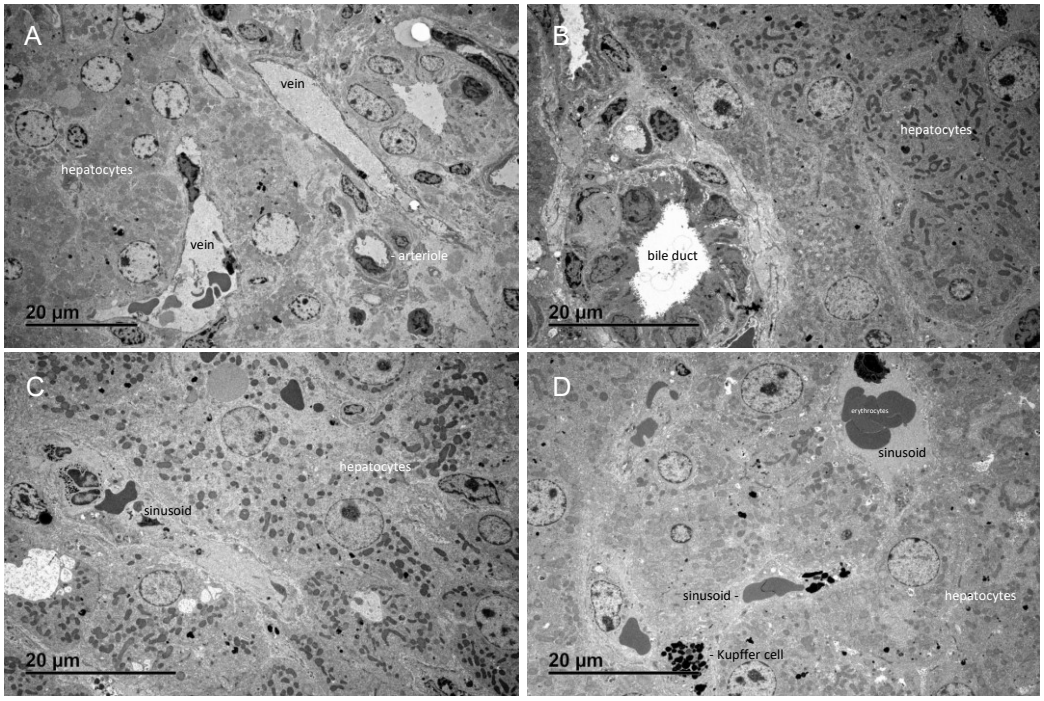
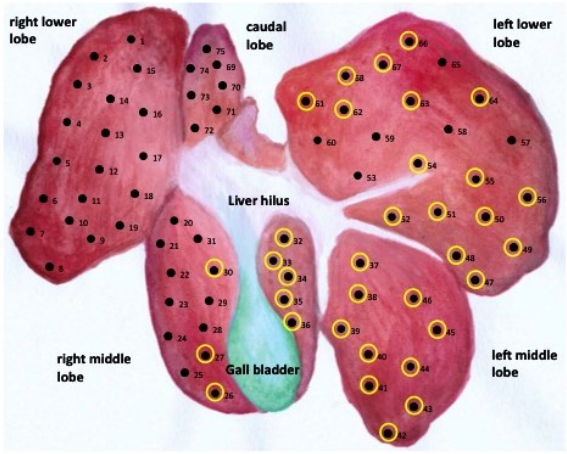
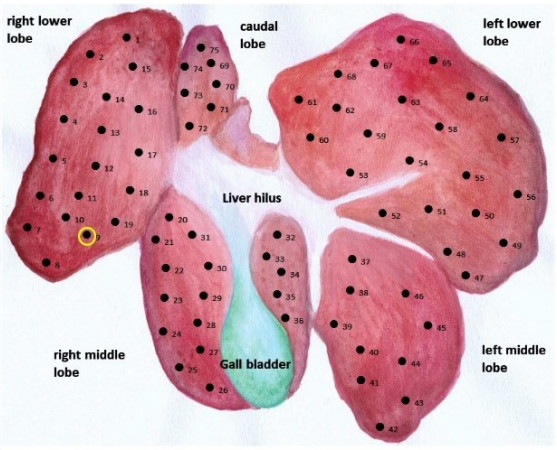


Figure 7: *TEM analyses of pig liver (pig A1) upon pressurized injection of two different conditions with sterile saline solution*. Injection conditions with either 30 ml/10sec or 100 ml/10sec followed by clamping of 1 minute were no morphological abnormalities visible. (A) Pre-injection, (B) upon 30 ml/10sec. (C) upon 100 ml/10sec. (D) overview of a hepatic triad (arteriole, vein and bile duct) upon 100 ml/10sec. Ultrastructurally, no alterations were observed in any of the specimens.

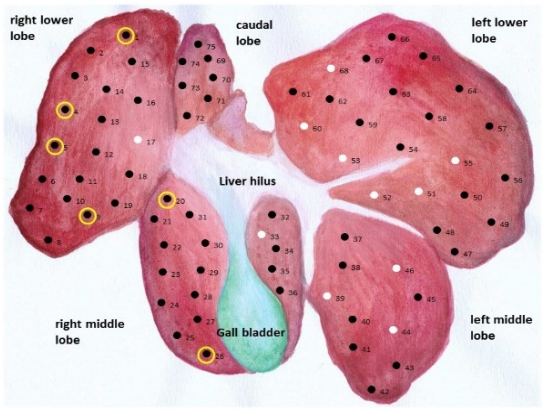
A B1, 12 mg, 6 h



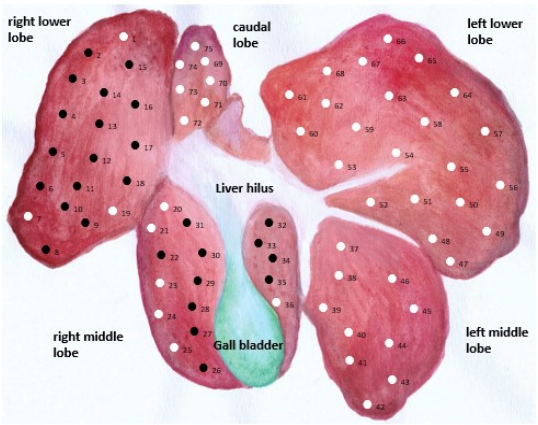
B B3, 12 mg, 3 d



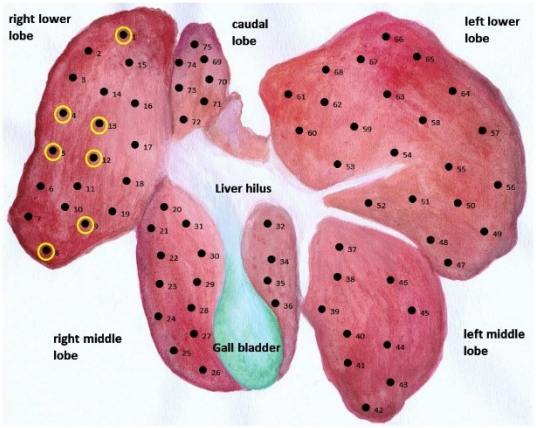
C B4, 2 mg, 10 d



D B5, 2 mg, 10 d



E B6, 12 mg, 10 d



F B7, 12 mg, 10 d

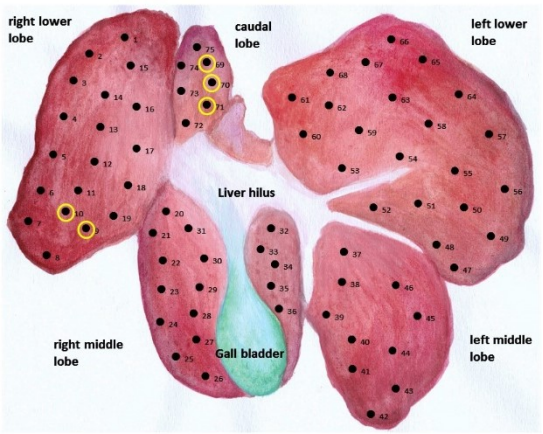


Figure 8: *Sketch of pig liver lobes of stable naked DNA (MC.P3-luc) delivery and luciferase expression upon hydrodynamic intrabiliary injection.* Drawing of a caudal view of a pig liver with position of collected samples indicated by black dots (PCR positive), white dots (PCR negative) and yellow circles (luciferase expression positive). Injection dosage in mg and time point of sacrifice in hours (h) or days (d) is mentioned. (**A**) **B1** (176-37): Injection of 12 mg naked DNA vector resulted in 100% transfection rate (75/75 samples) and a luciferase expression of 45% (34/75 samples). (**B**) **B3** (176-31): Injection of 12 mg naked DNA vector resulted in 100% transfection rate (75/75 samples) and a luciferase expression of 1.33% (1/75 samples). (**C**) **B4** (176-26): Injection of 2 mg naked DNA vector resulted in 85% transfection rate (63/75 samples) and 8% luciferase positive samples. (**D**) **B5** (176-28): Injection of unknown 2 mg naked DNA vector resulted in a transfection rate

of 36% (27/75 samples) but undetectable luciferase expression. (**E**) **B6** (176-29): Injection of 12 mg naked DNA vector resulted in 100% (74/74 samples) transfection rate and 9.45% luciferase expression (7/74 samples). (**F**) **B7** (176-30): Injection of 12 mg naked DNA vector showed a result of 100% (75/75 samples) transfection rate and a luciferase expression of 6.67% (5/75 samples).

Note that "10 days" is between 9 to 11 days after injection.

Table 3: Average of luciferase activity (RLU/µg protein; threshold 0.08 RLU/µg protein) and copy number (per diploid genome) of all collected samples for each lobe infused with vector MC.P3-luc.

	Pig B1 (12 mg; 6h)	**Pig B3** (12 mg, 3 d)	**Pig B4** (2 mg; 10 d)	**Pig B5** (2 mg; 10 d)	**Pig B6** (12 mg; 10 d)	**Pig B7** (12 mg; 10 d)
Liver Lobe						
Right middle						
Luciferase	0.10 ± 105	0.00 ± 0.00	0.05 ± 0.11	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.00
Vector genomes	5.43E+01 ± 105	2.16E-01 ± 0.25	7.04E-02 ± 0.06	9.12E-03 ± 0.01	5.40E-01 ± 0.49	3.36E-01 ± 0.34
Right lower						
Luciferase	0.03 ± 0.00	0.090 ± 0.02	0.03 ± 0.04	0.00 ± 0.00	0.04 ± 0.07	0.01 ± 0.04
Vector genomes	5.98E+01 ± 64	1.91E-01 ± 0.30	2.69E-01 ± 0.18	2.06E-03 ± 0.00	5.90E-02 ± 0.09	2.08E-01 ± 0.13
Left middle						
Luciferase	0.26 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.55	0.00 ± 0.00
Vector genomes	5.77E+01 ± 57	3.35E-01 ± 0.25	1.84E-03 ± 0.00	1.36E-02 ± 0.01	4.70E-01 ± 0.55	7.00E-01 ± 0.33
Left lower						
Luciferase	0.41 ± 0.00	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.00	0.20 ± 0.00	0.00 ± 0.00
Vector genomes	5.23E+02 ± 1767	2.66E-01 ± 0.20	9.21E-03 ± 0.01	1.28E-02 ± 0.01	2.01E-01 ± 0.11	2.53E-01 ± 0.15
Caudal						
Luciferase	0.25 ± 0.00	0.00 ± 0.00	0.01 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.06 ± 0.09
Vector genomes	1.14E+02 ± 128	9.65E-01 ± 0.05	2.36E-02 ± 0.02	2.92E-03 ± 0.00	6.26E-01 ± 0.01	6.01E-01 ± 0.54

4. Discussion

Establishment of hydrodynamic retrograde DNA vector administration via the bile duct in domestic weaned pigs was the main aim of this study. For this, we first established the surgical and hydrodynamic conditions including liver enzyme (AST and ALT) and tissue damage analyses (immunohistologcial stainings and TEM). The challenge of the intrabiliary method in small pigs was to keep the abdominal cavity clean of ingesta after enterotomy and of bile after catheterization of the major duodenal papilla. Optimal condition was found to be up to 12 mg of DNA vector in a volume of 100 ml/10sec., followed by 1 min clamping, without histological abnormalities in the liver. Despite the less mortality rate and the very well recovery of all piglets after surgery, the transfection rate and luciferase expression is weak compared to the intraportal injection.

This study was performed after successful hydrodynamic intrabiliary injections in mice (Deplazes et al., "Delivering Naked DNA to the Liver of Mice for Gene Therapy", in preparation) to establish a safe and feasible alternative to the hydrodynamic intraportal injection in large animals, in order to treat infants in future. We chose the pig due to similarity of size, anatomy and physiology to humans and according our experience with the intraportal injections in small pigs [17,30].

According, to following parameters (1) access, (2) safety and risk assessment, and (3) duration of surgical intervention we decided to access via the bile system in pigs. The surgical intervention is less invasive, easier and the surgical duration shorter than the portal vein injection. Hydrodynamic injections via the bile system have been described in rats and ERCP-guided in adult pigs but not in small pigs after weaning [18,19]. The purpose of both surgical techniques is to target specific periportal hepatocytes, since there are no expressed enzymes in hereditary liver metabolism in this area. The major difference in both techniques is that we target periportal hepatocytes on the one hand via the venous system and on the other hand via the bile system. The injected minicircle vector needs to be transported from the venous or bile system to the periportal liver cells. The mechanism is not clear but it seems that rapid infusion of large volume of naked DNA into the liver parenchyma induces the uptake and expression by some hepatocytes [8]. Efficient transfection in hepatocytes, confirmed by PCR, could be shown by hydrodynamic intrabiliary injection. As expected was a higher number of positive samples in pigs injected with 12 mg than with 2 mg of vector DNA seen. In comparison to the intraportal vein injection we observed, a shift of positive samples into the right instead of the left liver lobes [17]. This phenomenon can be explained by anatomical structures of the

porcine bile system and the catheter position. The position of the catheter was monitored by Real-Time XRay and held in position with clamps but it is still possible that the catheter tends to move to one side of the branch of the common bile duct during pressurized injection.
Low but stable luciferase activity was present in liver samples, except 176-28 (see above), after 10 days. Other publications saw high luciferase activity upon hydrodynamic injection at an earlier time point (4–24 hours after injection) this may explain the low luciferase activity after 10 days [15,24].

Additionally, we investigated in liver biopsies to rule out pressurized liver damage. Injections with two different flow rates conditions (30 ml in 10 sec and 100 ml in 10 sec.,) were performed, Histological staining confirmed no visible morphological cell damage after a flow rate of 100 ml in 10 sec or a 10-fold lower injection rate (30 ml in 10 sec, ***Figure 7*** and ***Figure 9***). In conclusion, of pressurized injections we could not observe any difference in the recovery of the animal either on the damage on hepatocytes. So lower injection conditions are also a possible option for HRII. No further conduct and studies in this study was done. Since we did not detect any washout of vector DNA into other tissue organs.
Based on our observations and compared to the results of the study by Stoller et. al 2014 higher mortality was observed in piglets with pressurized portal vein injections than with pressurized intrabiliary injections of MC.P3-luc vector. This technique is feasible, safe and less stressful in weaned pigs. The expression efficacy of the intrabiliary injection is much lower (10- to 100-fold) compared to the portal vein injection but can be improved on the vector side, including promoter, enhancer, codon-optimization, and replication with potentially higher efficacy and this could be an approach for an ERCP-guided access (see supplement).

5. Literature

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6. Supplement

Hydrodynamic intrabiliary injection of a next-generation nanovector (nSMARter.P3Luc1) to porcine hepatocytes

Introduction

Improvement of vector efficiency plays an important role in gene therapy. Luciferase expression with our current "standard" MC vector (MC.P3-luc) was always very low in pig liver upon intraportal injection but even lower upon intrabiliary infusion. Recently, we found that a novel non-integrating and autonomously replicating naked-DNA vector "nSMARter.P3Luc1" (not published; gift from Dr. Richard Harbottle, German Cancer Research Center, Heidelberg, Germany) exhibited a much higher and sustained luciferase expression in mice liver upon hydrodynamic (tail) vein infusion (HTV) (Deplazes et al., "Delivering Naked DNA to the Liver of Mice for Gene Therapy", in preparation). Another limitation of MC.P3-luc was the purification, including purity and the amount of DNA or yield, which is not the case for the novel vector as we received it pure. Furthermore, the pig liver almost doubled in total weight during the 10 days period between vector infusion and analysis (***Table 2***), which leads to vector dilution and eventually apparent lower luciferase expression. Based on this, we decided to perform the HRII procedure in total four (three pigs are shown C2–C4, the fourth pig was not used for further analyses due to liver rupture during infusion) pigs with 12 mg of the so-called next-generation nanovector nSMARter.P3Luc1 and analyzed the resected liver after 6 hours (n = 2) and 10 days (n = 2). We also included one animal with a lower flow rate (3 ml/sec instead of 10 ml/sec). The latter is based on other authors that recommended to infuse vector-DNA with a lower flow rate when injecting through the bile duct [18,25]. Furthermore, we wanted to investigate luciferase staining to verify transfection of hepatocytes (using a potentially more specific antibody, i.e. the goat anti-luciferase polyclonal) as described by others [24].

Material and Methods

Animal Handling

See page 8, section 2. Material and Methods.
Three-weeks-old domestic pigs (weight 4.3–6.7 kg; ***Table 2***) were separated from their mothers after weaning and brought to a loose barn with porcine mates 5 days before surgery.

Anaesthesia and postoperative monitoring

See pages 10ff, section 2. Material and Methods. Animals were kept for 6 hours or 10 days.

Surgical procedures

See pages 11ff, section 2. Material and Methods.

Collection of samples

See pages 14ff, section 2. Material and Methods.

Nanovector/nSMARter.P3Luc1

We used a purified nSMARter.P3Luc1 (4644bp) vector, containing the firefly luciferase gene controlled by a liver-specific-promoter P3, a replicating element scaffold matrix attachment region (SMAR) and an isolator Ele40. It was purified and provided by the laboratory of Dr. Richard Harbottle, German Cancer Research Center, Heidelberg, Germany.

Copy number assay

See page 16, section 2. Material & Methods.

Luciferase activity

See page 17, section 2. Material & Methods.

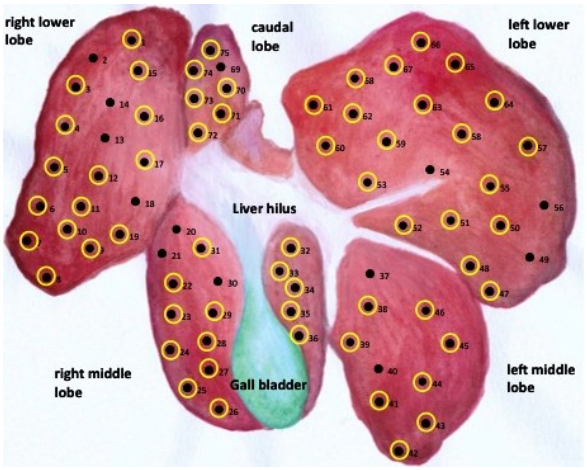
Results and Discussion

The successful study with the naked DNA vector lead us to try the procedure with a novel non-integrating and autonomously replicating naked-DNA vector "nSMARter.P3Luc1". This next generation vector nSMARter.P3Luc1 showed a 100% transfection rate in all pigs (C2–C4) and a significant and equal distribution of 85.3% luciferase expression upon hydrodynamic intrabiliary injection of 12 mg vector DNA after 6 hours with following conditions 100 ml/10 sec. (***Figure 9*****A**). Higher luciferase expression was expected, as similar results are with the naked DNA vector. 10 days after injection under the same conditions the luciferase expression is low, in both pigs C3 and C4 (***Figure 9*****B and C**). One explanation of the low expression could be that pigs grow that fast that the vector is "washed out" during liver growth. Another study showed higher luciferase gene expression in pigs with a body weight of 40 – 45 kg with sequential injections to multiple liver lobes at an interval of 30 min. Six weeks later at a body weight of 60 kg the injections were repeated [15,24]. Here, the pig is not growing that fast as a weaned piglet; second, they injected to multiple liver lobes and repeated the injection set with a balloon catheter to prevent backflow. The distribution more to the right side of the liver is due to catheter placement and anatomy of the pig liver. An overview of the average of luciferase activity and the copy number of all pigs (C2 – C4) can be seen in (***Table 4***). Here, further investigations in immunohistological stainings were performed to detect luciferase positive hepatocytes on cryo-sections. No histological damage to the liver tissue was observed (data not shown). It is very difficult to distinguish a positive reaction

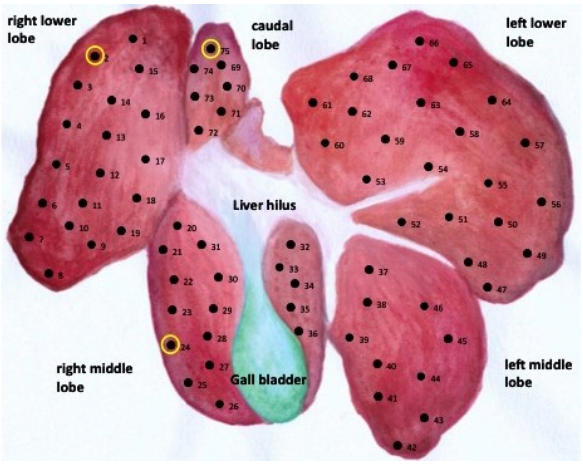
from hepatocellular bile and hemosiderin because the staining is always brown. Therefore, AEC (3-Amino-9-ethylcarbazol) chromogen was used for better demonstration of luciferase positive reaction to distinguish from hepatocellular bile and hemosiderin. Hepatocellular bile and hemosiderin is a more crystal shiny brown compared to hepatocytes (mat brown). Positive reaction could be observed focal and nuclear in hepatocytes (***Figure 10***).

This study enabled us to have a deeper look into the impact upon hydrodynamic intraportal injection with a novel vector. Histologically could not any tissue damaged be detected, independent of pressure. Under the developed conditions, all piglets show a homogenous distribution over all liver lobes of PCR positive samples. Not surprisingly is a higher detectable luciferase activity 6 hours upon injection, what is also described in several studies [15,24]. To optimize the luciferase activity sequential target multilobar injections can be discussed to be more efficient but is probably difficult and not feasible in weaned piglets due to body size. Further studies are needed to optimize the luciferase expression in weaned piglets.

A C2, 12 mg, 6 h



B C3, 12 mg, 10 d



C C4, 12 mg, 10 d

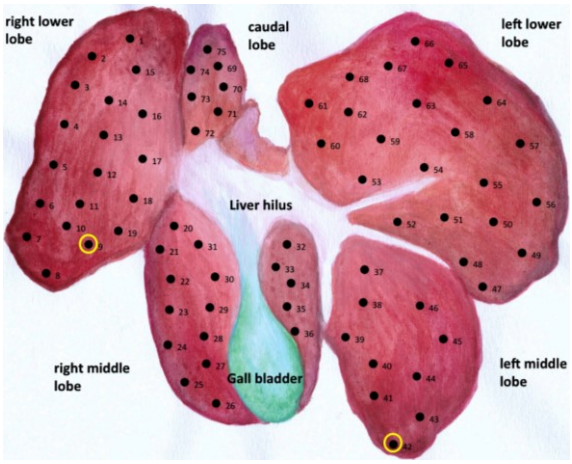


Figure 9*: Overview of pig liver lobes of stable naked DNA (nSMARter.P3luc1) delivery and luciferase expression upon HRII*. Position of collected samples is indicated by dots; black dots are PCR positive, white dots PCR negative, and yellow circles are positive luciferase expression. Injection dosage in mg and time point of sacrifice in hours (h) or days is mentioned. **(A) C2** (176-36): Injection of 12 mg naked DNA vector resulted in an equal luciferase expression of 85.3% (64/75 samples positive) with a flow rate of 100 ml/10sec. after 6 hours. **(B) C3** (176-32): Injection of 12 mg naked DNA vector resulted in a luciferase expression of 4.00 % (3/75 samples positive) with a flow rate of 100 ml/10sec. after 10 days. **(C) C4** (176-33): Injection of 12 mg naked DNA vector resulted in a luciferase expression of 2.67% (2/75 samples positive) with a reduced flow rate of 30 ml/10sec. after 10 days. A sample was positive above the threshold of 0.08 RLU/µg protein. Note, "10 days" is between 9 to 11 days after injection.

Table 4: Average of luciferase activity (RLU/µg protein; threshold 0.08 RLU/µg protein) and copy number (per diploid genome) of all collected samples for each lobe infused with vector nSMARter.P3.luc1.

	Pig C2 (12 mg; 6h)	**Pig C3** (12 mg, 10 d)	**Pig C4** (12 mg; 10 d)
Liver Lobe			
Right middle			
Luciferase	0.38 ± 0.32	0.00 ± 0.02	0.02 ± 0.01
Vector genomes	6.83E+02 ± 1402.70	6.38E+00 ± 2.39	1.01E+01 ± 3.18
Right lower			
Luciferase	0.34 ± 0.27	0.01 ± 0.02	0.04 ± 0.08
Vector genomes	5.57E+01 ± 53.88	4.91E+00 ± 1.88	8.43E+00 ± 2.29
Left middle			
Luciferase	0.21 ± 0.17	0.00 ± 0.00	0.02 ± 0.03
Vector genomes	4.40E+02 ± 426.57	8.43E+00 ± 2.83	8.88E+00 ± 1.99
Left lower			
Luciferase	0.41 ± 0.31	0.00 ± 0.00	0.01 ± 0.02
Vector genomes	1.03E+02 ± 114.15	8.73E+00 ± 3.12	1.02E+01 ± 1.88
Caudal			
Luciferase	0.25 ± 0.16	0.04 ± 0.09	0.01 ± 0.01
Vector genomes	1.56 E+02 ± 303.19	8.43E+00 ± 3.78	1.03E+01 ± 2.34

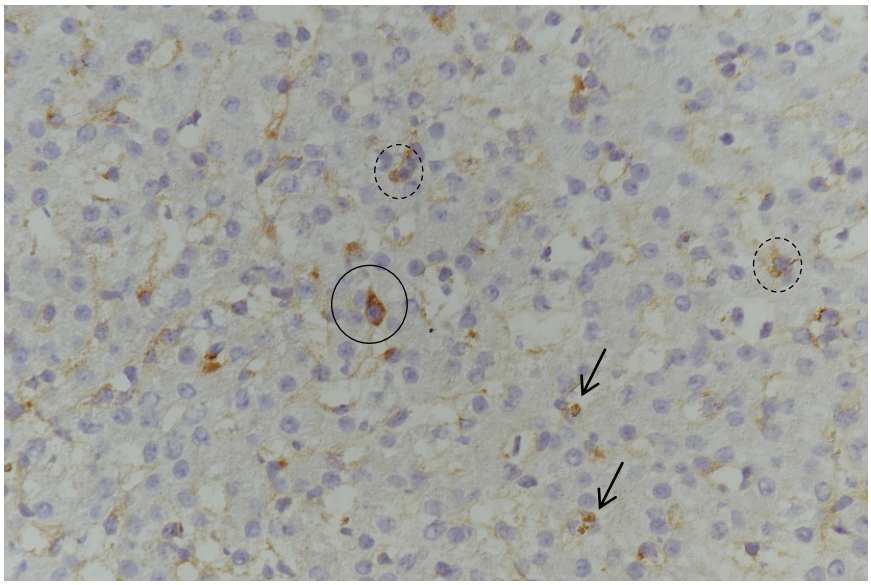


Figure 10: *Cryo sections and formalin fixed paraffin embedded pig liver (Ffpe) for Luciferase detection.* Focal positive cytoplasmatic (black circle) and nuclear immune reaction (dashed circles) AEC chromogen. AEC chromogen shows hemosiderin or bile (= brown shiny pigment, black arrows). AEC (3-Amino-9-ethylcarbazol) chromogen.

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8. Curriculum Vitae

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